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Relaxin Stimulates Leukocyte Adhesion and Migration through a Relaxin Receptor LGR7-dependent Mechanism*[S]

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Leukocytes are critical effectors of inflammation and tumor biology. Chemokine-like factors produced by such inflammatory sites are key mediators of tumor growth that activate leukocytic recruitment and tumor infiltration and suppress immune surveillance. Here we report that the endocrine peptide hormone, relaxin, is a regulator of leukocyte biology with properties important in recruitment to sites of inflammation. This study uses the human monocytic cell line THP-1 and normal human peripheral blood mononuclear cells to define a novel role for relaxin in regulation of leukocyte adhesion and migration. Our studies indicate that relaxin promotes adenylate cyclase activation, substrate adhesion, and migratory capacity of mononuclear leukocytes through a relaxin receptor LGR7-dependent mechanism. Relaxin-stimulated cAMP accumulation was observed to occur primarily in non-adherent cells. Relaxin stimulation results in increased substrate adhesion and increased migratory activity of leukocytes. In addition, relaxin-stimulated substrate adhesion resulted in enhanced chemotaxis to monocyte chemoattractant protein-1. These responses in THP-1 and peripheral blood mononuclear cells are relaxin dose-dependent and proportional to cAMP accumulation. We further demonstrate that LGR7 is critical for mediating these biological responses by use of RNA interference lentiviral short hairpin constructs. In summary, we provide evidence that relaxin is a novel leukocyte stimulatory agent with properties affecting adhesion and chemomigration.

Complex interactions between tumor cells and the immune system regulate disease progression by stimulating cell proliferation, neovascularization, tissue remodeling, and metastasis or by inhibiting the host anti-tumor immune response (1). However, the factors produced by tumor cells that affect their immune surveillance remain to be fully elucidated. The insulin-related peptide hormone, relaxin, possesses key features required of a tumor-derived factor capable of affecting malignant progression, and its emerging role as a putative mediator of tumor progression has been recently reviewed (2). In humans, relaxin is encoded by three genes designated H1, H2, and H3 (3). Functionally, relaxin is classically described to improve blood supply to multiple organs including the uterus, mammary gland, lung, and heart (4). Relaxin is also known to increase matrix metalloproteinase expression, resulting in collagen turnover of reproductive tissues and in models of fibrosis, and is responsible for lengthening of the interpubic ligament during delivery (3–5).

A role for relaxin in cancer progression was first suggested 38 years ago by studies indicating that carcinogen-fed rats experienced substantially enhanced mammary tumor growth when co-treated with relaxin several weeks later (6). More recently, relaxin has been shown to support growth and invasiveness of breast cancer cells (7, 8), and elevated relaxin serum levels are positively correlated with breast cancer metastases (9). Relaxin immunostaining has been demonstrated in epithelial and myoepithelial cells of normal and cancerous breast tissue (10), and significantly higher levels of relaxin staining occur in neoplastic breast tissues as compared with normal breast tissue (11). Similarly, relaxin is produced by the normal prostate (12–16), and expressed in prostate cancer cell lines LNCaP, DU145, and PC3 (17, 18). Relaxin has been implicated in increased tumor growth and angiogenesis of PC3 prostate xenografts (19), whereas we have found that H2 relaxin expression is up-regulated in LNCaP cells undergoing neuroendocrine differentiation (20). Together, these studies strongly implicate relaxin in breast and prostate cancer progression. Relaxin has been attributed with regulation of several intermediary responses or intracellular signaling events in a variety of cell types. These include cAMP, protein kinase A (21–27), mitogen-activated protein kinase, cAMP-response element-binding protein (28), tyrosine phosphorylation (22, 29), nitric-oxide synthase activation (30–38), and calcium influx (39). These intermediary signaling events are then implicated in the up-regulation of matrix metalloproteinases (29, 40–43), epidermal growth factor (44), insulin-like growth factor (45, 46), insulin-like growth factor-binding proteins (47), vascular endothelial growth factor (28, 43, 48), and guanylyl cyclase activation (32, 33, 35). In inflammatory wound sites relaxin also induces vascular endothelial growth factor and basic fibroblast growth factor production in macrophages (48). Prevailing questions are whether the ability of tumors to secrete relaxin affects the surrounding stromal and endothelial cells or hematopoietic cells and whether relaxin can stimulate and/or recruit these cells to produce factors that promote tumor survival and growth.

Here we demonstrate that relaxin activates adenylate cyclase, promotes substrate adhesion, and proportionally enhances migratory capacity of mononuclear leukocytes in a relaxin receptor LGR7-dependent mechanism. Relaxin-stimulated cAMP accumulation occurs primarily in non-adherent cells, and this responsiveness is lost once the cells have adhered to a substrate. Relaxin alone can promote migration of leukocytes and can also enhance their monocyte chemoattractant protein-1 (MCP-1)[2] migratory response. These enhanced migratory responses may be a result of relaxin-mediated substrate adhesion in

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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[2] The abbreviations used are: MCP-1, monocyte chemoattractant protein 1; PBMC, peripheral blood mononuclear cells; RLXH, histidine$_6$-tagged recombinant human H2 relaxin; FSK, forskolin; IBMX, isobutylmethylxanthine; TBP, TATA-binding protein; PMA, phorbol 12-myristate 13-acetate; GF109203X, broad spectrum protein kinase C inhibitor; shRNA, short hairpin interfering RNA.

these cells. We also describe for the first time that relaxin can dose-dependently stimulate cAMP accumulation in peripheral blood mononuclear cells (PBMC) and that this response is proportional to relaxin-stimulated adhesion and migratory activity. We further demonstrate that LGR7 is critical for mediating these biological responses by use of RNA interference lentiviral short hairpin constructs. These results implicate relaxin as a novel stimulator of leukocyte recruitment.

EXPERIMENTAL PROCEDURES

Cell Culture—PBMC were isolated from fresh whole blood obtained from healthy male and female volunteers by density gradient sedimentation in Histopaque (Sigma) according to the manufacturer's instructions. PBMC were washed twice in phosphate-buffered saline, resuspended in RPMI (Invitrogen), and used immediately in subsequent assays. THP-1 cells (ATCC) were cultured in RPMI and 10% fetal bovine serum. All THP-1 cell experiments were performed with cells cultured less than 15 passages that were serum-starved overnight before treatments.

Production of Recombinant THP-1 Clonal Populations—Stably infected THP-1 populations were generated by lentiviral infection, stable integration, and expression of short hairpin interfering RNA (shRNA) sequence targeting LGR7 (LGR7 GenBank^{TM} accession number NM_021634). shRNA target sequences of LGR7 were selected according to previously established guidelines (49, 50), and complementary oligonucleotides were synthesized, annealed, and ligated into the pSHAG-1 vector backbone (Cold Spring Harbor Laboratory) (49). Recombination reactions (Gateway, Invitrogen) were performed to transfer the target sequences into the Gateway-modified pHR-CMV-eGFP lentiviral vector (51). 10 μg of this recombinant vector was co-transfected with 7.5 μg of the packaging plasmid pCMV-gag-pol (ΔR8.2) and 2.5 μg of the VSV-G-expressing envelope plasmid pMD.G into HEK293T cells (51–53) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Virus-containing conditioned media was collected every 24 h for 2 days, filtered through 2.0-μm pores (Millipore), and immediately used to infect THP-1 cells, which were subsequently sorted by flow cytometry to obtain the enhanced green fluorescent protein tag expression approaching 100%. Of the six shRNA targets created (Supplemental Fig. 1), two, L7–274 (5′-CACCTGAATGTTTGGTCGGTTCTGTGCCAG-3′) and L7–917 (5′-GATTGAAAATCTTCCACCGCTTATATTCAA-3′), which effectively suppressed LGR7 expression in THP-1 cells, are described in these studies.

cAMP Assays—5×10^5 cells were treated with 6 His-tagged recombinant human H2 relaxin (RLXH) (20) at 0 and 0.0013, 0.0025, 0.005, 0.01, 0.02, or 0.04 ng/ml or forskolin (FSK, 10 μM) and isobutylmethylxanthine (IBMX, 50 μM) for 30 min (relaxin H2 GenBank^{TM} accession number NM_134441). To control for the RLXH vehicle, all samples were treated with the volumetric equivalent of the nickel column elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, and 0.05% Tween 20, pH 8.0) as previously described (20). Non-adhered cells were collected from the media by centrifugation and resuspended in lysis buffer (Biotrak ELISA kit, Amersham Biosciences). For analysis of the total population cAMP response, the remaining adhered cells were lysed by pooling with the lysis of the corresponding non-adhered cells. For analysis of THP-1 subpopulations, adhered and non-adhered cells were collected as above and passively lysed separately. Adenylate cyclase activation was measured by competitive cAMP enzyme-linked immunosorbent assay (Biotrak ELISA kit, Amersham Biosciences) and normalized to cell equivalents by protein content (BCA assay, Pierce) according to the manufacturer's instructions and expressed as fmol of cAMP/μg of cellular protein.

Cell Adhesion and Migration Assays—For adhesion assays, 5×10^5 cells were seeded into wells of 24-well tissue culture dishes and left untreated or treated with RLXH (0.02 ng/ml) or phorbol 12-myristate 13-acetate (PMA, 50 ng/ml) and incubated for 24 h at 37 °C. Non-adhered cells were removed by decanting and washing the plates three times with RPMI media. For migration assays, 5×10^5 cells were seeded in the upper chamber of 24-well 5-μm transwell cell culture inserts (Costar). Cells were treated with RLXH (0.02 ng/ml), GF109203X (2 μM), FSK (10 μM), and/or MCP-1 (10, 25, or 50 ng/ml). All samples not treated with RLXH received an equivalent volume of the RLXH vehicle unless otherwise stated. Migration on uncoated transwells was performed for 24 h at 37 °C, and migration on laminin-coated transwells was performed for 3 h at 37 °C. The number of adhered or migrated cells was determined by CyQuant DNA binding fluorescence (C-7026, Molecular Probes, Invitrogen) (54). Relative fluorescence was detected at 485-nm excitation and 527-nm emission by plate reading fluorometer (Fluoroskan Accent FL, ThermoLabsystems) and standardized for cell number. Total and percent adhered or migrated cells were calculated for each assay, and pooled results are expressed as adhesion index or migration index, respectively, normalized to cell number of untreated controls.

Real Time PCR Analysis—LGR7 mRNA expression of parental and shRNA-expressing THP-1 cells was determined from cells under normal culture conditions. Total RNA was isolated using TRIzol reagent (Invitrogen), DNase-treated (Invitrogen) and used to generate single-stranded cDNAs using Superscript first-strand synthesis system (Invitrogen) according to the manufacturer's instructions. RNA (2 μg) was primed for cDNA synthesis using a random hexamer primer (0.5 μg/μl). The comparative C_T (threshold cycle) method was used to measure LGR7 mRNA levels under different treatment conditions using a GeneAmp 5700 Sequence detection system and the GeneAmp 5700 SDS software (Applied Biosystems). The LGR7 primers LGR7-Rfp (5′-CCCCTGTGGGAACATCACA-3′) and LGR7-Rrp (5′-GTTGTCCT-CATCGGCCTGATT-3′) and probe (5′-VIC-CTCCTGCACTGTA-ACGGTGTGGACGACT-TAMRA-3′) were selected using the Primer Express software Version 1.5 (Applied Biosystems). The probe was labeled with a VIC/TAMRA quencher/reporter. A region of TATA-binding protein (TBP) cDNA was amplified using control primer/probe kits from Applied Biosystems. Primer verification analysis was performed to ensure the LGR7/TBP primer pairs had equal amplification efficiencies (data not shown). TBP mRNA was unaffected by lentivirus infection or shRNA expression and was, therefore, deemed a suitable control for normalizing the results to total mRNA levels. The real time PCR reactions were performed using TaqMan Universal PCR Master Mix (Applied Biosystems) such that the LGR7 and TBP base-line C_T (threshold cycle) ranges were 5–22 and 5–20, respectively. The threshold log values used in the LGR7 and TBP analyses were 0.03–0.05.

Fluorescence-activated Cytometric Analysis—5×10^5 live cells suspended in 200 μl of phosphate-buffered saline were stained for cell surface LGR7 expression by incubation with 5 μg/ml rabbit polyclonal anti-LGR7 antibody (ab12714, Abcam) for 45 min at 4 °C. To correct for nonspecific IgG background binding to cell surface, a parallel cell aliquot was stained with a rabbit IgG antibody against the intracellular protein, Akt (anti-Akt, 9272, Cell Signaling). After washing with phosphate-buffered saline, cells were treated with a 1:200 dilution of donkey anti-rabbit R-phycoerythrin-conjugated antibody (Jackson Immunoresearch) for 30 min at 4 °C. Cells were washed twice with phosphate-buffered saline and then immediately analyzed on flow cytometer (Epics

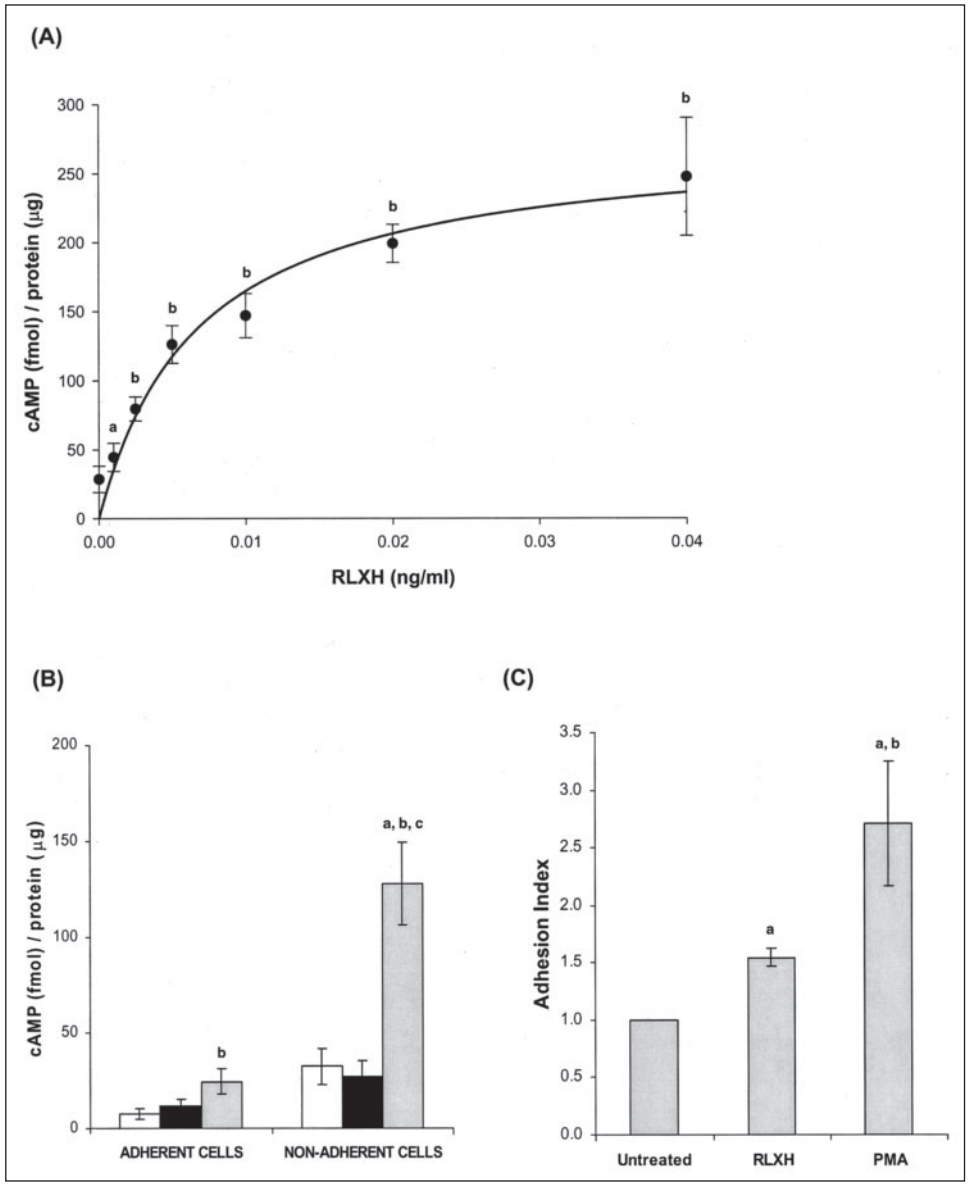

FIGURE 1. **Relaxin stimulates adenylate cyclase activation and substrate adhesion of THP-1 cells.** *A*, serum-starved THP-1 cells were plated into a 24-well tissue culture plate followed immediately by treatment with RLXH (0 and 0.0013, 0.0025, 0.005, 0.01, 0.02, or 0.04 ng/ml) and incubated at 37 °C for 30 min. Non-adhered and adhered cells were lysed together in the 24-well plate. Adenylate cyclase activation was expressed as fmol of intracellular cAMP normalized to μg of whole cell lysate protein. Values represent the means ± S.E. of five independent experiments, with each sample assayed in quadruplicate. Paired *t* test analyses: *a*, *versus* untreated $p < 0.05$; *b*, *versus* untreated $p < 0.001$. *B*, adenylate cyclase activation in adhered and non-adhered THP-1 cells was determined in cells treated as in *A* except that non-adhered cells were collected and assayed separately from adhered cells. Non-adhered cells accounted for 65% of the total cell number as estimated by relative whole cell lysate protein. *White bars*, untreated; *black bars*, Mock; *gray bars*, RLXH. Values represent the means ± S.E. of six independent experiments assayed in triplicate. Paired *t* test analyses: *a*, *versus* respective mock treatment, $p < 0.05$; *b*, *versus* respective untreated, $p < 0.05$; *c*, *versus* RLXH-treated adhered cells, $p < 0.05$. *C*, relaxin-stimulated substrate adhesion of THP-1 cells was measured in cells treated with PMA (50 ng/ml), RLXH (0.02 ng/ml), or untreated (vehicle alone) and incubated at 37 °C for 24 h as described under "Experimental Procedures." Substrate adhesion index was calculated as a –fold change of CyQuant fluorescence normalized to that of the untreated samples of washed wells. Values represent the means ± S.E. of eight independent experiments assayed in triplicate. Paired *t* test analyses: *a*, *versus* untreated $p < 0.01$; *b*, *versus* RLXH treatment $p < 0.05$.

xl-mcl, Beckman Coulter) with Expo32 software (Beckman Coulter) and WinMDI software.

Statistical Analysis—Curve fit analysis was performed using SigmaPlot graphics software (SPSS) for linear regression (Fig. 2*B*) or nonlinear, one-site saturation kinetics (Figs. 1*A* and 3*A*). Statistical analysis was performed with JMPIN statistical discovery software (SAS Institute). Significant differences among treatments were identified by one-way analysis of variance, and significant differences between multiple pairs of treatments were identified by multi-sample matched paired t-tests analyses at a threshold of $p < 0.05$ unless otherwise noted in the figure legends.

RESULTS

Relaxin Activates Adenylate Cyclase Preferentially in Non-adherent THP-1 Cells—To assess the capacity of relaxin to affect leukocyte activity, we have generated RLXH (20). Bioactivity of this reagent purified by nickel column chromatography was assessed by determining its ability to stimulate adenylate cyclase activity in target cells. We previously demonstrated that RLXH stimulates adenylate cyclase activation in LGR7-expressing HEK 293T cells (20). Here we observed that RLXH stimulates a dose-dependent increase in intracellular cAMP levels in the monocyte/macrophage cell line THP-1 with first order saturation kinetics ($r^2 = 0.9614$). Intracellular cAMP levels were significantly elevated above basal levels by as little as 1.3 pg/ml RLXH and saturated at 9-fold over basal levels (276.9 fmol of cAMP/μg of protein) with a calculated half-maximal dose of 6.8 (±1.8) pg/ml RLXH 30 min after stimulation (Fig. 1*A*). These results are consistent with previous observations for relaxin-mediated adenylate cyclase activation of THP-1 cells (21, 22, 24) and confirm that RLXH is a biologically active relaxin source.

THP-1 cells in culture are in equilibrium between adherent and non-adherent cells, which may represent phenotypic differences with respect to differentiation or activation state and relaxin responsiveness. We, therefore, assessed potential differences in adenylate cyclase activation by RLXH in adherent and non-adherent populations. Although the non-adherent THP-1 monocyte populations accounted for 65% of the total cell number, they were responsible for 85% of the cAMP response to RLXH (Fig. 1*B*). This comparison suggests that non-adherent THP-1 cells respond preferentially to relaxin and that this relaxin-

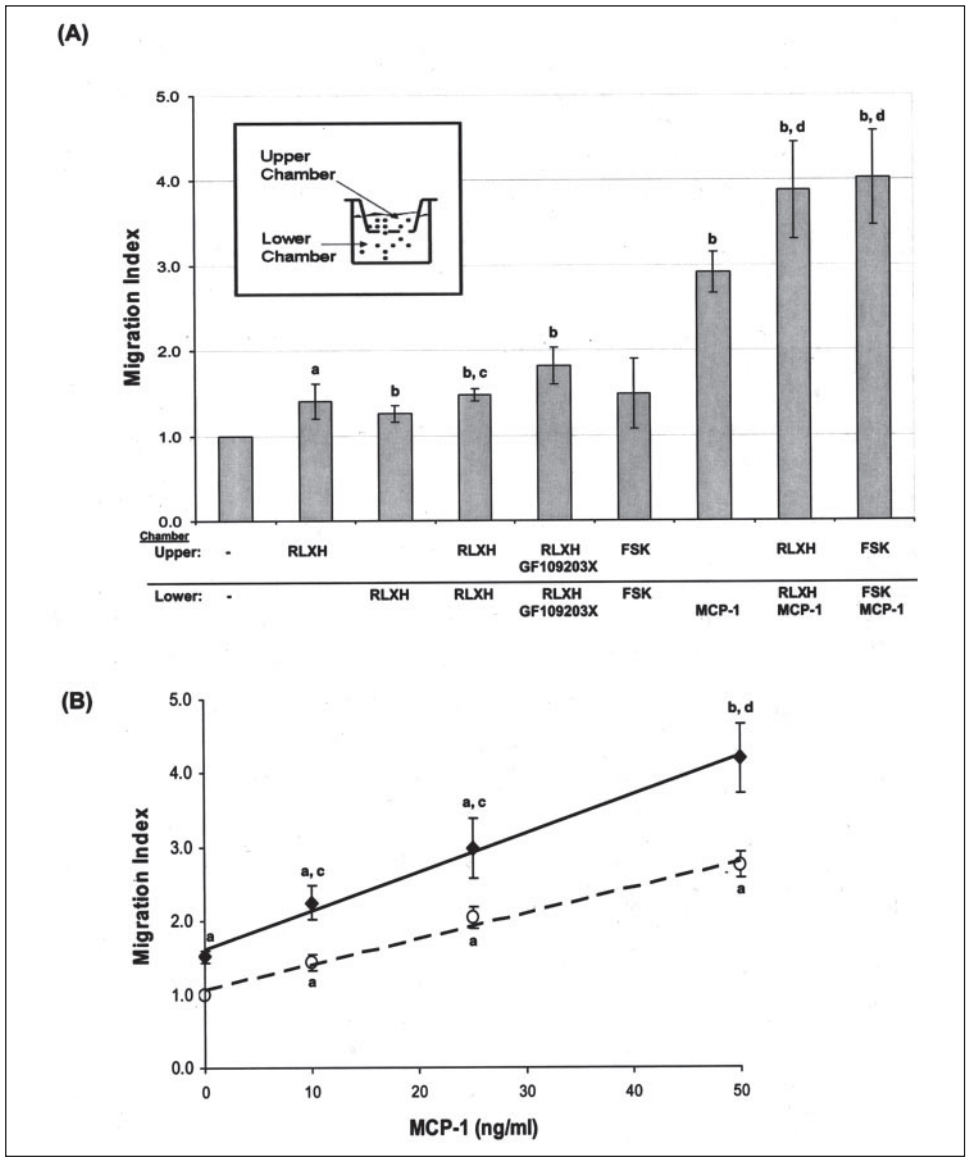

FIGURE 2. **Relaxin stimulates migration of THP-1 cells.** After overnight serum starvation, THP-1 cells were seeded in the upper chamber of 24-well 5-μm pore transwells followed immediately by treatment. *A*, migration into the lower chamber of the transwell of untreated cells was compared with that of cells exposed to RLXH (0.02 ng/ml) added to the upper, the lower, or both chambers (relaxin checkerboard analysis). Migration of cells treated with RLXH (0.02 ng/ml) in both chambers were compared with RLXH-treated cells in the presence of GF109203X (2 μM) or FSK (10 μM) in both chambers. THP-1 migration was also assessed for cells treated with MCP-1 (50 ng/ml) in the lower chamber in the absence or presence of either RLXH (0.02 ng/ml) or FSK (10 μM) in both chambers. All samples were incubated at 37 °C for 24 h. The migration index was calculated as a –fold change of relative CyQuant fluorescence of cells migrated into the lower chamber normalized to that of the untreated samples (vehicle alone (−)). Values represent means ± S.E. of six independent experiments assayed in triplicate. Paired *t* test analyses: *a*, *versus* untreated, $p < 0.05$; *b*, *versus* untreated, $p < 0.01$; *c*, *versus* RLXH in lower chamber, $p < 0.05$; *d*, *versus* MCP-1 in lower chamber, $p < 0.01$. *B*, the effect of relaxin treatment on the MCP-1 chemotactic response was compared in THP-1 cells stimulated with MCP-1 in the lower chamber at (0 and 10, 25, or 50 ng/ml) in the absence (*dashed line*) or presence (*solid line*) of RLXH (0.02 ng/ml) in both chambers. The migration index was calculated as above, and values are expressed as the means ± S.E. from 10 independent experiments assayed in triplicate. Paired *t* test analyses: *a*, *versus* untreated, $p < 0.01$; *b*, *versus* untreated, $p < 0.001$; *c*, *versus* no RLXH at same MCP-1 dose, $p < 0.05$; *d*, *versus* no RLXH at same MCP-1 dose, $p < 0.01$.

induced adenylate cyclase response may be attenuated upon substrate adhesion.

Relaxin Stimulates Substrate Adhesion of THP-1 Cells—Although the ability of THP-1 cells to respond to relaxin through activation of adenylate cyclase and other signaling pathways has been well characterized (21, 22, 24, 28, 48), any physiological consequence of relaxin stimulation has remained undetermined. Based on the above observation, we speculated that relaxin may promote substrate adhesion of non-adherent monocytic cells. To examine whether relaxin can promote adhesion of monocytes to tissue culture plastic, untreated THP-1 cells were compared with cells treated with either RLXH or PMA, an agent well known to promote THP-1 adhesion and macrophage differentiation (55). In a temporal analysis comparing the number of adherent cells in untreated and RLXH-stimulated THP-1 cultures, no significant difference in cell adhesion was observed before 8 h (data not shown). At 24 h RLXH induced a 1.5-fold increase in THP-1 adhesion above basal levels (from 30 to 45%), whereas PMA stimulated the expected robust adhesion of 80% of the monocytic population (Fig. 1*C*). These results indicate that relaxin has the previously uncharacterized property of stimulating monocyte substrate adhesion.

Relaxin Stimulates Migration of THP-1 Cells—We reasoned that an increased ability of monocytes to adhere to surfaces may promote or enhance migratory capacity through transwells in Boyden-chamber based assays (56, 57). Temporal analysis of THP-1 migration using a transwell migration assay showed no difference in RLXH-induced migratory kinetics before 8 h of stimulation (data not shown), but an increased number of migrated cells could be observed at longer times, which was optimal at 24 h after stimulation (1.5-fold, Fig. 2*A*). To determine whether RLXH can induce directional migration, a checkerboard analysis was performed in which THP-1 cells seeded in the upper chamber were exposed to RLXH in either the lower or upper chamber or in both chambers (Fig. 2*A*). At 24 h after treatment, THP-1 cell migration into the lower chamber was significantly increased in all three RLXH treatment conditions. THP-1 cells exposed to the greatest concentration of RLXH (0.02 ng/ml in both chambers) demonstrated an increased propensity to migrate to the lower chamber (30% of the seeded population), 1.5-fold greater than the untreated control (20%). Similarly, migration of cells exposed to RLXH (0.02 ng/ml) when added to only the upper (27%) or lower chamber (25%) was enhanced over untreated controls but were indistinguishable from one another. Cells exposed to

RLXH added to only the lower chamber exhibited a reduced migratory capacity compared with cells exposed to RLXH in both chambers, likely because of the reduced initial local concentration of RLXH when it is sourced farther away from the seeding location. These data demonstrate that although relaxin alone cannot induce directional (chemotactic) migration, it is capable of promoting dose-dependent chemomigration proportional to its capacity to induce cAMP accumulation and adhesion.

Relaxin Augments Migration of THP-1 Cells toward MCP-1—Because of the numerous reports demonstrating that relaxin induced activation of the adenylate cyclase signaling in multiple cell types (21–27), we assessed whether this pathway was important in mediating the RLXH-stimulated migratory response in THP-1 cells. We determined that relaxin-induced migration can be replicated by the addition of the adenylate cyclase activator, FSK, and that relaxin-induced migration is unaffected by the broad spectrum protein kinase C inhibitor, GF109203X (Fig. 2*A*). This suggests that relaxin likely induces chemomigration through a cAMP-dependent and protein kinase C-independent pathway. The archetype MCP-1 (58–60) was added to the lower chamber to confirm the chemotactic responsiveness of THP-1 cells (Fig. 2*A*). At 24 h, MCP-1 increased migration of THP-1 cells into the lower chamber almost 3-fold such that 50% of the cells had migrated into the lower chamber. Intriguingly, we also observed that MCP-1 chemotaxis was significantly enhanced when RLXH was added to both chambers. In the presence of RLXH, MCP-1 chemoattraction was increased to almost 4-fold over basal levels, such that 75% of THP-1 cells had migrated into the lower chamber. This RLXH-induced augmentation of MCP-1 chemotaxis was precisely mimicked when MCP-1 treated cells were co-stimulated with FSK (Fig. 2*A*). These results indicate that relaxin-induced adenylate cyclase activation provides an important alternative pathway to enhance monocyte responsiveness to the protein kinase C-dependent MCP-1 chemotactic response (61, 62).

To further investigate the capacity of relaxin to augment MCP-1-mediated chemomigration, we compared the migration of THP-1 cells to varying concentrations of MCP-1 in the presence or absence of RLXH (Fig. 2*B*). THP-1 cell migration was linearly responsive to stimulation with 0–50 ng/ml MCP-1 ($r^2 = 0.9943$, y intercept = 1.0684 ($\pm$0.0747) migration index (MI), slope = 0.0347 ($\pm$0.0026) MI/ng/ml MCP-1). We observed that RLXH co-stimulation proportionally increased THP-1 cell migration by 1.52-fold ($\pm$0.0227) over that of cells treated with MCP-1 alone at all MCP-1 concentrations tested ($r^2 = 0.9966$, y intercept = 1.6268 ($\pm$0.0869) migration index (MI), slope = 0.0521 ($\pm$0.0031) MI/ng/ml MCP-1). The consequence of relaxin exposure is that the chemotactic response at maximal MCP-1 dose (50 ng/ml) can be replicated at 2.5 times lower dose of MCP-1 (20 ng/ml) in the presence of RLXH (0.02 ng/ml in both chambers). These results support the notion that relaxin may make the cells more responsive to MCP-1 at lower doses and that the presence of relaxin can modulate leukocytic chemomigration toward limiting concentration of chemoattractants such as MCP-1. The cAMP-signaling dependent adhesion response of the cells to relaxin is complementary to the protein kinase C-dependent chemomigratory response of the cells to MCP-1, which may explain the additive augmentation of migration observed in RLXH- and MCP-1-stimulated cells.

Relaxin Activates Adenylate Cyclase, Promotes Adhesion, and Enhances Migration of Peripheral Blood Mononuclear Cells—To examine the physiological relevance of these experiments in the context of leukocyte biology, we assessed whether relaxin could regulate similar responses in PBMC. Although maximal relaxin-induced adenylate cyclase activity in PBMC was about 15% that observed in comparably treated THP-1 cells (saturating at 36.72 fmol of cAMP/μg of protein), it nonetheless mirrored the first order dose-dependent saturation kinetics of RLXH-stimulated THP-1 cells ($r^2 = 0.8782$). Intracellular cAMP levels in RLXH-stimulated PBMC were significantly elevated above basal levels by as little as 2.5 pg/ml RLXH and saturated at 5-fold over basal levels. The calculated half-maximal dose for PBMC of 3.6 ($\pm$1.7) pg/ml RLXH at 30 min after stimulation (Fig. 3*A*) was statistically indistinguishable from the corresponding half-maximal RLXH dose calculated for THP-1 cells described for Fig. 1*A*.

Also consistent with the response of THP-1 cells, at 24 h RLXH induced a 1.5-fold increase in adhesion of PBMC to a tissue culture plastic substrate (from 55 to 85%) that was proportional to their response to PMA (Fig. 3*B*). Furthermore, relaxin induced a 1.8-fold increase in the migratory response of PBMC (from 20 to 35% of seeded cells; Fig. 3*C*). This response was approximately half the migratory response induced by MCP-1 (60% of seeded cells) and is in excellent agreement with our previous observations in THP-1 cells. Together these data suggest that relaxin is a key mediator of adenylate cyclase activation, substrate adhesion, and migratory responses of PBMC.

Short Hairpin Targeting Suppresses LGR7 mRNA and Protein Levels in THP-1 Cells—To verify that the relaxin receptor, LGR7 (63), was responsible for mediating relaxin-regulated adenylate cyclase and chemomigration responses of THP-1 cells, we developed an RNA interference approach to inhibit LGR7 expression using lentiviral infection, stable integration, and expression of short hairpin interfering RNA sequence independently targeting six different regions of LGR7 (Supplemental Fig. 1). Of these six targeted sequences, two proved to be effective in down-regulating LGR7 expression at the mRNA and protein levels (Figs. 4, *A* and *B*, respectively). Using real time PCR analysis, we found that LGR7 mRNA expression was substantially impaired (3-fold) in cells expressing shRNA targeting LGR7 sequences starting at nucleotides 274 and 917 (cell lines designated L7–274 and L7–917, respectively). Control THP-1 cell lines expressing 2- and 4-base pair mismatches of the 274 sequence (mm2 L7–274 and mm4 L7–274, respectively) both exhibited LGR7 mRNA levels that were indistinguishable from that of wild type THP-1 cells (Fig. 4*A*). To validate target suppression at the protein level, we performed fluorescence-activated cytometric analysis of live cells using an LGR7 antibody targeting the amino-terminal extracellular domain (Fig. 4*B*). After correcting for negative control cell surface staining, cell surface LGR7 expression in L7–274 cells was 20% that observed in wild type THP-1 cells, whereas LGR7 expression in L7–917 cells was 40% that of wild type THP-1 cells. In both the mismatch L7–274 cell clones, mean LGR7 expression was about 70% that of wild type THP-1 cell levels. Although the distribution of the fluorescence-activated cytometric analysis data suggests that there is still substantial overlap of surface expression levels between the various THP-1 cell populations, these results are consistent with shRNA-mediated suppression of mRNA expression and suggest that the LGR7 transcript and protein expression is substantially impaired in L7–917 and L7–274 cells and is relatively intact in the mismatch L7–274 cell clones.

LGR7 Is Required for Relaxin-regulated Adenylate Cyclase Activation in THP-1 Cells—Next, we examined whether suppressing LGR7 expression affected relaxin-stimulated adenylate cyclase activation (Fig. 5*A*). To determine whether the adenylate cyclase function in L7–274 cells was still intact, FSK and IBMX were used to stimulate intracellular cAMP production. FSK- and IBMX-stimulated adenylate cyclase activation in L7–274 cells was indistinguishable from that generated by wild type THP-1 cells. However, L7–274 exhibited a dramatic attenuation of cAMP accumulation relative to wild type THP-1 cells upon stimulation

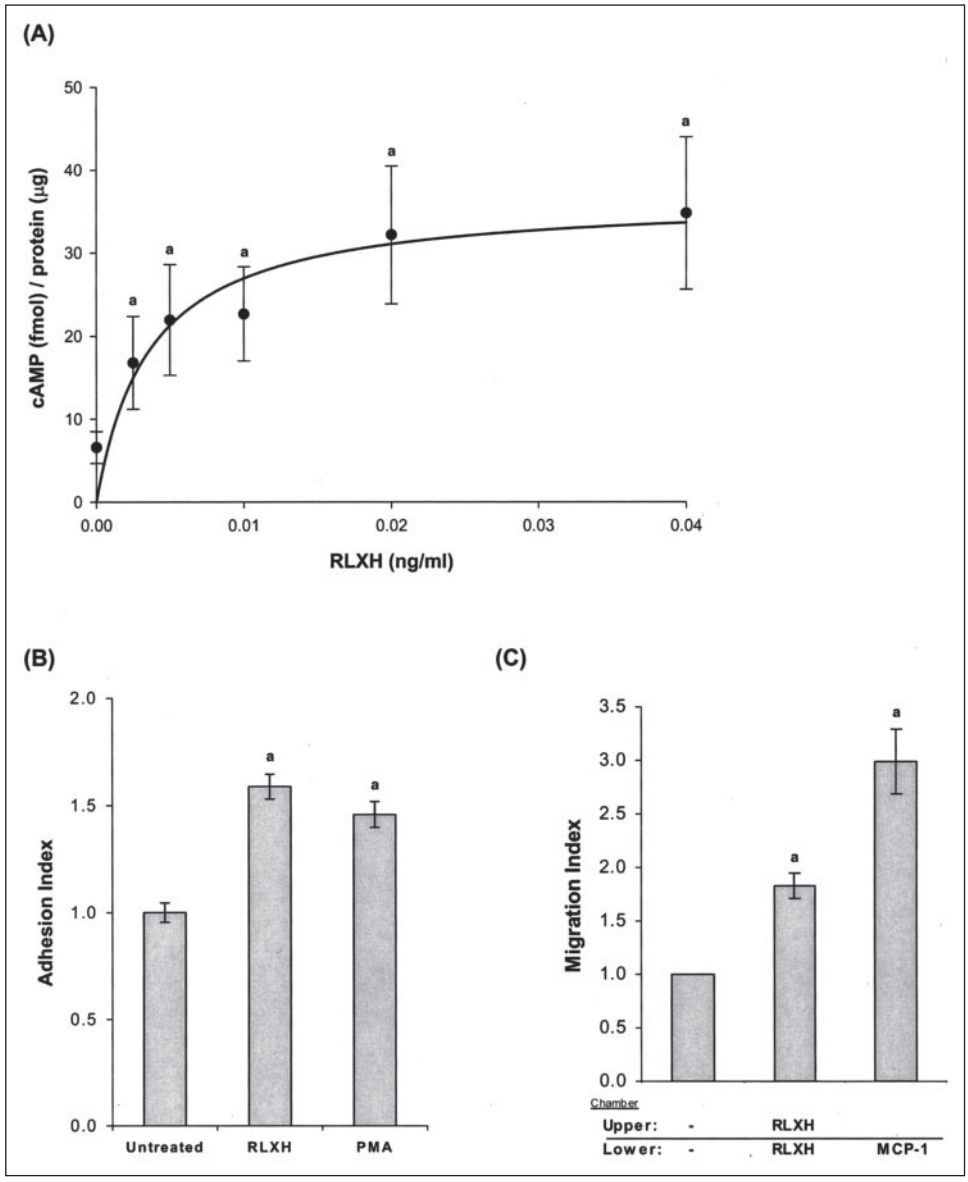

FIGURE 3. **Relaxin regulates peripheral blood mononuclear cell biology.** *A*, relaxin-stimulated activation of cAMP production in PBMC was measured in cells treated with RLXH (0 and 0.0025, 0.005, 0.01, 0.02, or 0.04 ng/ml) at 37 °C for 30 min. Adenylate cyclase activation is measured as fmol of cAMP normalized to μg of whole cell lysate protein. Values represent means ± S.E. of 3–5 independent experiments assayed in triplicate. Paired *t* test analyses: *a*, *versus* untreated, $p < 0.05$. *B*, for measuring relaxin-stimulated adhesion of PBMC to tissue culture treated-plastic, cells were simultaneously plated and treated with RLXH (0.02 ng/ml), PMA (50 ng/ml), or untreated (vehicle alone) and incubated at 37 °C for 24 h as described under "Experimental Procedures." Substrate adhesion index was calculated as -fold change of CyQuant fluorescence normalized to that of the untreated samples of washed wells. Values represent the means ± S.E. of three technical replicates. Paired *t* test analysis: *a*, *versus* untreated, $p < 0.05$. *C*, to measure relaxin-stimulated migration of PBMC, cells were seeded in the upper chamber of 24-well 5-μm transwells followed immediately by treatment with either RLXH (0.02 ng/ml), vehicle alone (−), or MCP-1 (50 ng/ml without vehicle) at 37 °C for 24 h. Cells that migrated to the lower chamber were measured by CyQuant fluorescence, and the migration index was calculated as –fold change of fluorescence normalized to the untreated samples. Values represent means ± S.E. from six independent experiments assayed in triplicate. Paired *t* test analyses: *a*, *versus* untreated $p < 0.01$.

with RLXH. L7–917 cells also exhibit a suppressed response to RLXH. When exposed to relaxin, mm2 L7–274 cells show an intermediate cAMP response, and mm4 L7–274 cells exhibit a response not significantly different from wild type THP-1 cells. This suggests that lentiviral infection of THP-1 cells does not in itself impede their ability to respond to relaxin and that the targeted suppression of LGR7 is likely proportional to the specificity of the shRNA sequence. Together, these results indicate that suppression of LGR7 levels in L7–274 cells blocks their ability to respond to RLXH and that this suppressed response is specific to relaxin since the FSK- and IBMX-induced adenylate cyclase response remains unaffected. These data provide evidence that relaxin stimulates cAMP accumulation in THP-1 cells through an LGR7-dependent signaling pathway and that the LGR7 receptor is essential for initiating this signal.

LGR7 Is Required for Relaxin-regulated Migratory Potential in THP-1 Cells—To determine whether the relaxin-induced migratory response of THP-1 cells is mediated by LGR7, we compared the migratory responses of wild type THP-1 cells with L7–274 and mm4 L7–274 cells. To demonstrate the chemokine-like properties of the relaxin peptide at a more acute time point, THP-1 wild type and recombinant cells were assessed for migratory responses to relaxin under conditions that were more conducive for such activity. As previously established, monocytes migrate favorably on laminin-coated transwells (64). When THP-1 migratory response was assessed at 3 h on laminin-coated transwells, wild type cells exhibited responses to RLXH, MCP-1, and both treatments which were essentially identical to those observed at 24 h on uncoated transwells (*i.e.* 1.5-, 3-, and 4-fold, respectively, compared with untreated) (Fig. 5*B* *versus* Fig. 2*A*). We observed a substantial impairment of the relaxin-augmented MCP-1 chemotactic response in L7–274 cells migrating across laminin-coated transwells that was not observed in the mm4 L7–274 control cells (Fig. 5*B*). These data demonstrate that the relaxin-regulated migratory responses can occur under more acute timelines when provided with the appropriate substrate and that the LGR7 signaling pathway is crucial for mediating such responses.

DISCUSSION

Intercellular communication between tissue at a site of inflammation and distal blood cells relies on production of diffusible molecular mediators. Tumor cells exist in inflammatory microenvironments because

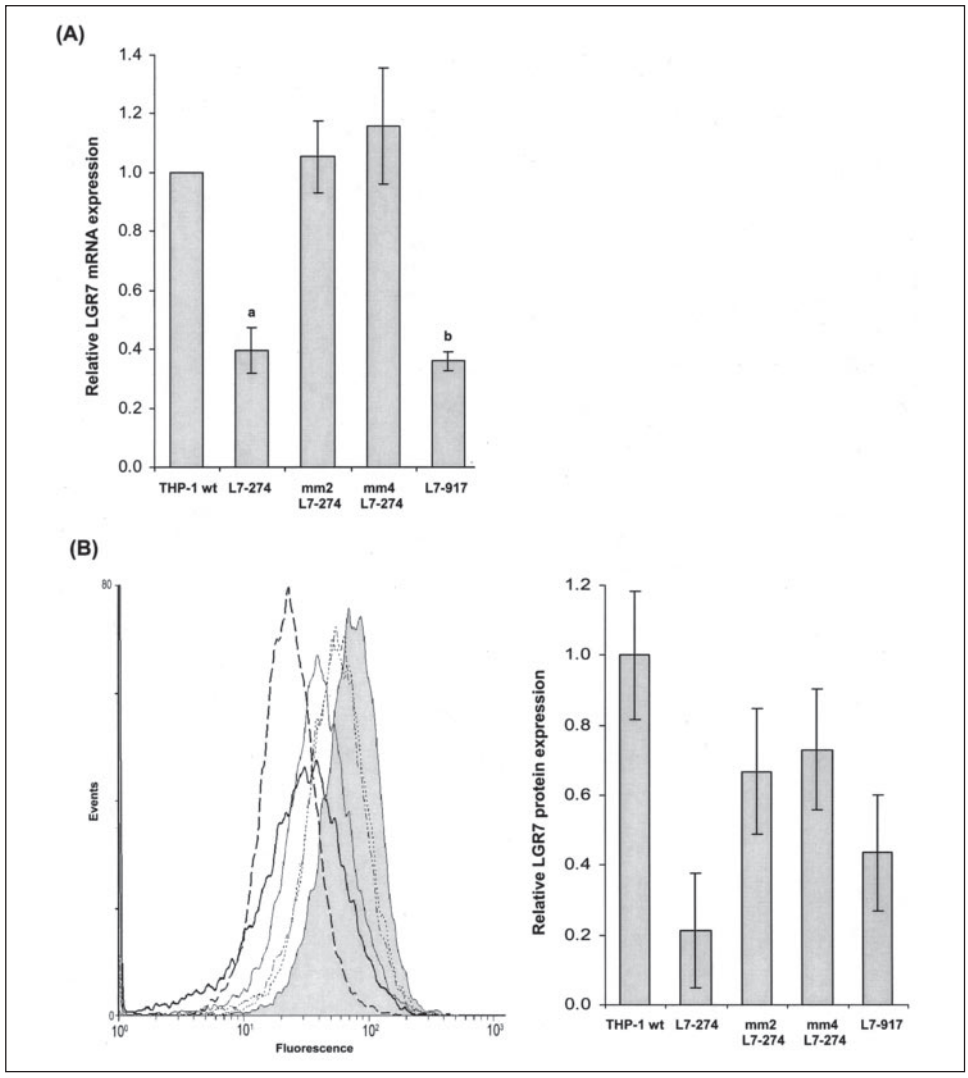

FIGURE 4. **shRNA suppression of LGR7 expression in recombinant THP-1 cells.** *A*, total RNA was extracted from THP-1 wild type, L7–274, mm2 L7–274, mm4 L7–274, and L7–917 cells under normal culture conditions. Real time PCR analysis was performed using LGR7 and TBP primer/probe pairs. LGR7 mRNA levels were normalized to TBP mRNA levels, and all values are expressed relative to parental (*wt*) THP-1 cells. Values represent the means ± S.E. of three independent experiments assayed in triplicate. Paired *t* test analyses: *a*, *versus* THP-1 wild type, $p < 0.05$; *b*, *versus* THP-1 wild type, $p < 0.01$. *B*, LGR7 protein expression was determined by flow cytometry analysis of live cells stained with polyclonal anti-LGR7 antibody and a secondary anti-rabbit IgG coupled to a phycoerythrin fluorophore. Control cells were incubated with an anti-Akt antibody and the phycoerythrin-coupled secondary antibody. Other controls, unstained or stained with secondary antibody alone, showed low background fluorescence (not shown). A representative fluorescence-activated cytometric analysis plot is shown. *Shaded area*, THP-1 wild type; *solid thick line*, L7-274; *dashed thin line*, mm2 L7-274; *dotted thin line*, mm4 L7-274; *solid thin line*, L7-917 cells. A representative αAkt signal in wild type cells is shown (*dashed thick line*). Relative LGR7 protein expression for each cell type is calculated by internally correcting for background non specific staining of intracellular anti-Akt antibody and expressed as the mean relative fluorescence intensity ± S.D. normalized to wild type THP-1 cells.

they emulate wounds that will not heal (65). Tumor cells must be able to manipulate their microenvironment to support their growth, and this includes recruitment and functional alteration of immune cells from surveillance toward assistance (1). In order for this to occur, tumor-derived chemokines must first stimulate the target immune cells circulating in the bloodstream. In this report, we assessed relaxin-induced stimulation of two THP-1 cell populations by comparing acute adenylate cyclase responses to RLXH in non-adherent and adherent populations and found that non-adherent cells account for most of the cAMP response to relaxin (Fig. 1*B*). This indicates that non-adherent cells are the major relaxin-responsive population and suggests that once cells have adhered, this relaxin-regulated response may no longer be necessary.

This prompted us to examine whether relaxin can directly promote adhesion of leukocytes and whether this might be correlated with an enhanced migratory capacity. Here, we show that relaxin induces a 1.5-fold enhancement in THP-1 cell adhesion that is identical to the relaxin-induced 1.5-fold enhanced migration and the 1.5-fold augmentation of MCP-1-induced migration (Figs. 1*C* and 2, *A* and *B*). These responses were proportional to relaxin-induced cAMP accumulation and could in part be replicated by direct adenylate cyclase activation (Figs. 1*A* and 2*A*). We also confirmed that relaxin similarly regulates cAMP production, adhesion, and migratory responses of PBMC (Fig. 3, *A*–*C*). Hence, relaxin-induced migratory responses of leukocytes may be dependent on relaxin-stimulated substrate adhesion through a cAMP-dependent mechanism. These relatively modest changes of relaxin-regulated migratory response are substantially quicker when laminin-coated transwells are provided as an adhesion and migration substrate (Fig. 5*B* *versus* 2*A*), suggesting that relaxin-mediated effects are substrate dependent. It is possible that these effects could be accentuated further in the presence of other relevant substrates.

Adhesion is an essential step required for leukocytic migration, and modest changes to *in vitro* cell migration have previously been shown to have substantial biological effects. As an example, RANKL was shown to induce a 1.6-fold enhancement of monocyte migration (66) and a 1.3-fold enhancement of PBMC migration (67). This relatively modest effect of RANKL on monocyte migration is correlated with substantial biological relevance (68, 69). Adhesion of leukocytes to vascular endothelium require several signals from chemokine-like molecules that in turn regulate the migratory response (70). For example, MCP-1 and interleukin-8 can each independently induce adhesion of monocytes to vascular endothelium (71). In addition, the adhesion interaction between leukocytes and endothelium stimulates production of leukocyte-derived cytokines/chemokines, which serve to further activate the immune response (72). This suggests that relaxin-mediated adhesion may also modulate similar immune functions.

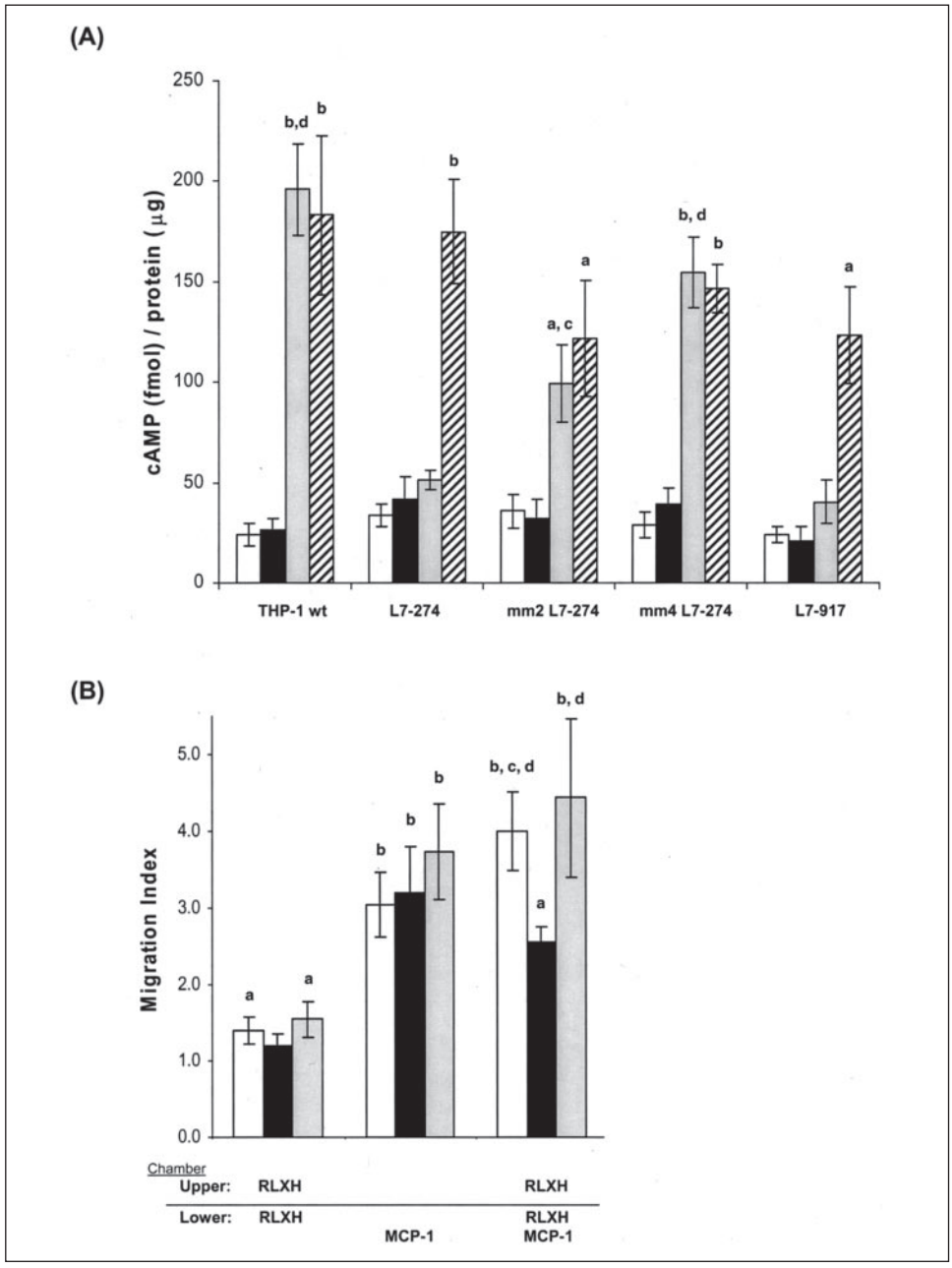

FIGURE 5. **Suppression of LGR7 expression impairs relaxin-induced adenylate cyclase activation and migration of THP-1 cells.** *A*, THP-1 wild type, L7–274, mm2 L7–274, mm4 L7–274, and L7–917 cells were tested for cAMP responsiveness to RLXH. Serum-starved cells were seeded into wells of 24-well plate and left untreated (*white bars*) or treated with either, mock (vehicle control) (*black bars*), RLXH (0.02 ng/ml) (*gray bars*), or FSK (10 μM) and IBMX (50 μM) (*hatched bars*) at 37 °C for 30 min. Adenylate cyclase activation was measured as fmol of cAMP normalized to μg of whole cell lysate protein. Values represent the means ± S.E. of six independent experiments assayed in triplicate. Paired *t* test analyses: *a*, *versus* respective untreated and mock, $p < 0.05$; *b*, *versus* respective untreated and mock, $p < 0.01$; *c*, *versus* L7–274 RLXH treatment, $p < 0.05$; *d*, *versus* L7–274 RLXH treatment, $p < 0.01$. *B*, impairment of relaxin-induced migratory response by LGR7 suppression in THP-1 cells was assessed in serum-starved cells seeded in the upper chamber of 24-well 5-μm pore transwells precoated with laminin (10 μg/ml). Cells were left untreated (not shown), treated with RLXH (0.02 ng/ml) in both chambers, or stimulated with MCP-1 (50 ng/ml) in the lower chamber in the absence or presence of RLXH (0.02 ng/ml) in both chambers at 37 °C for 3 h. THP-1 wild type (*white bars*), L7–274 (*black bars*), mm4 L7–274 cells (*gray bars*). The number of migrated cells was determined by CyQuant fluorescence, and the migration index was calculated as –fold change of fluorescence compared with untreated cells. Values represent means ± S.E. of four independent experiments assayed in triplicate. Paired *t* test analyses: *a*, *versus* untreated, $p < 0.05$; *b*, *versus* untreated, $p < 0.01$; *c*, *versus* wt MCP-1 treated in the lower chamber, $p < 0.05$; *d*, *versus* L7–274 same treatment, $p < 0.05$.

Although the current study is the first to demonstrate stimulatory migration effects of relaxin in mononuclear leukocytes, a number of studies have reported inhibitory functions for relaxin through a nitric oxide-dependent pathway in mast cells (30), platelets (32), and the polymorphonuclear leukocytes neutrophils (37) and basophils (38). Although relaxin has no effect on polymorphonuclear eosinophil invasion of the rat cervix during pregnancy (73), other studies have reported that relaxin positively regulates the migratory capacity of epithelial cells. Relaxin can stimulate migration and invasion of human breast cancer cells MCF-7 and SK-BR3 on matrigel-coated transwells (8). Similarly, relaxin can stimulate migration of canine breast cancer cells CF33.Mt on laminin-coated transwells, although this effect cannot be replicated on uncoated transwells (74). Relaxin can also accelerate migration of bronchial epithelial cells in a wound healing assay (75). Such studies indicate that relaxin-regulated migratory responses are not restricted to cells of a single lineage.

Cytokines and hormones are diffusible molecular mediator peptides that are defined by the cells upon which they act. Chemokines were originally defined as groups of cytokines that specifically target subsets of blood cells for chemoattraction. It is clear now that many of these same molecules can elicit similar chemoattraction responses in all cell types including epithelial carcinomas (1, 76). Occasionally the distinction between a cytokine and a hormone becomes vague or redundant since some of these can act on both cell types. The current study demonstrates for the first time that the classic hormonal role for relaxin as a regulator of epithelial and stromal functions (4) can now be expanded to include a role in targeting blood cells as a cytokine and possibly a chemokine. Our data depict a unique role for relaxin in positive regulation of cells of the immune system.

A significant enhancement of MCP-1 migratory response is observed in the presence of relaxin in THP-1 cells and PBMC on uncoated transwells and in THP-1 cells on laminin-coated transwells. This suggests

that relaxin can rapidly activate the adhesion-dependent migratory response under appropriate substrate conditions. This is the first demonstration of relaxin-regulated migration through transwell inserts on any cell type in less than 24 h. Furthermore, the enhanced MCP-1 regulated migratory response in the presence of relaxin is additive at all concentrations of MCP-1 tested (Fig. 2*B*). Based on these results, we suggest that by directing substrate adhesion, relaxin can augment cellular response to chemoattractants such as MCP-1 at limiting concentrations and that relaxin may act in concert with MCP-1 and perhaps other chemokines to enhance recruitment and infiltration of mononuclear cells to relaxin-producing tumor sites. Interestingly, MCP-1 expression has not been detected in prostate adenocarcinomas (77), although its expression is substantially elevated in breast adenocarcinomas (78, 79). Because relaxin is expressed in both of these adenocarcinomas, it is possible that relaxin fulfills an MCP-1 related role in these cancers. In the milieu of prostate and breast inflammatory tumor tissue, relaxin is likely secreted in combination with other cytokines and chemokines to regulate recruitment and infiltration of the mononuclear cells.

To verify that the relaxin receptor LGR7 is required to mediate these relaxin-regulated biological responses in monocytes, recombinant THP-1 cells in which LGR7 expression was suppressed were generated by expression of interfering RNA targeting of LGR7 (Fig. 4, *A* and *B*). We have determined that relaxin-mediated cAMP production and migratory activity are dependent on LGR7 activation because both biological responses are lost in L7–274 and intact in mismatch cell lines (Fig. 5, *A* and *B*). From our results that the migratory response to relaxin is unaffected by inhibition of protein kinase Cs, we suggest that protein kinase C is not required to propagate this relaxin-stimulated migratory response (Fig. 2*A*). The relaxin-mediated chemomigration response and the relaxin-enhanced MCP-1 response require activation of the G-protein coupled relaxin receptor (LGR7), and these responses can be replicated by FSK-induced adenylate cyclase activation. This implies that a LGR7/Gαs/adenylate cyclase/cAMP pathway is necessary to regulate the relaxin-induced migratory response.

Although further studies are required to examine the role of relaxin in leukocyte extravasation and infiltration of the tumor, the current study does show that relaxin has a definitive role in recruitment of leukocytes. Because tumor-infiltrating leukocytes often promote cancer in response to chemokinetic factors derived from the tumor itself, the presence of a relaxin gradient emanating from a tumor site may be an indicator of a poor prognosis for cancer patients. To provide molecular targets for selective inhibitors and activators, future studies will require elucidation of specific fate commitment and differentiation pathways induced by relaxin.

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